V. Summary and Conclusions

Functional characterization of master regulatory genes in plants as well as animals has elucidated many mechanistic aspects of morphogenesis Yet, in most instances the details of molecular mechanisms triggering activation of genes required for tissue differentiation and the control of cell proliferation are not known The ABCDE model of flower development suggests the combined effects of regulatory factors serves to activate specific differentiation programs in each floral whorl The cumulative effect is organ patterning, through regulated cell division and entry into differentiation programs Many of the regulatory ABCDE floral organs patterning factors encode conserved MADS-domain containing transcription factors, a subset of which, function as homeotic selector genes Mutations conferring loss-of-function or gain-of-function alter floral organ patterns creating novel floral morphologies The large-scale morphological variations observed in flowers may arise from functional diversification and duplication of few ancient conserved regulators Investigations on monocot-dicot MADS box genes lend support to these predictions Grass flowers are highly-divergent and are borne on branched inflorescences in complex structures with several bracts surrounding the reduced flower The grass floret organs lemma, palea and lodicules are structurally distinct therefore studies of grass homologs for dicot ABCDE floral organ patterning genes can illustrate the role of evolutionarily conserved function as opposed to divergent species-specific functions for the MADS gene family Molecular phylogeny identifies *OsMADS1* as a grass-specific member related to eudicot *SEP* family members Such studies also find that duplicated MADS-box genes contribute to class B and class C functions in grasses.

V.1 *Functions for the grass-specific OsMADS1 in the differentiation of specific cell types in lemma and palea and its role on inner whorl organ fate*

Our analysis identifies *OsMADS1* and its grass-specific relatives as factors, related to the eu-dicot *SEP* family These genes are predicted to have co-evolved with diversification of grasses, thereby implicating a unique role for these genes in grass flower organ development. Functional characterization of these genes, in transgenic plants over-expressing and knockdown for *OsMADS1*, was done in an independent study in the laboratory, to provide support for this prediction These studies had grossly examined

effects of ectopic expression *OsMADS1* and found empty glumes to be converted into lemma/palea like-organs, while the knockdown of *OsMADS1* affected lemma development coupled with the formation of glume-like in all whorls The rice florets had defects in floral determinacy upon knockdown of *OsMADS1* (Prasad et al , 2001, Prasad et al , 2005) The specific effect of *OsMADS1* on differentiation of cell-types typical of the lemma and palea was analysed here Firstly, while lemma and palea appear superficially similar we have identified distinguishing epidermal cellular features for these organs Scanning electron microscopy and histology reveals increased epidermal cell numbers, as a consequence of *OsMADS1* over-expression in the transformed lemma-like glume Further, even in the lemma, a floret organ that normally expresses OsMADS1, increased epidermal cell differentiation is seen The homeotically transformed lemma-like glume acquires sclerenchymatous and parenchymatous cells implying that *OsMADS1* alone can transform these spikelet organs into lemma We find complementary effects on epidermal cell proliferation/differentiation in florets with knockdown of *OsMADS1* These data indicate *OsMADS1* functions as an upstream regulator of genes controlling proliferation in the epidermis, and for genes triggering differentiation of sclerenchymatous and spongy parenchymatous cells within these grass-specific floral organs. A regulatory effect for *OsMADS1* in specifying all floret organs is evident from the failure of organ pattering in third whorl where glume-like cell are found in place of the lodicules These data demonstrate that *OsMADS1* functions-like a grass-specific regulator, consistent with its evolutionary diversification during the large-scale emergence of grasses

To provide mechanistic clues for OsMADS1 action we identified genes whose expression depends on *OsMADS1* Differential display RT-PCR and subtractive hybridizations, was used to isolate several cDNA, expressed differentially in either wild-type inflorescences from lines with *OsMADS1* RNAi based knockdown Some of these expressed sequences were cloned and the detailed computational sequence analysis for forty of these potential downstream genes was performed. A significant fraction of forward subtracted clones (i.e expressed in dsRNAiOsM1 inflorescence) are structural proteins, enzymes or hypothetical proteins, while many of the reverse-subtracted clones (i e expressed in wild-type inflorescences) are predicted transcription factors, putative signalling molecules and polyproteins. Amongst these differentially expressed factors, a

rice *GH3*-like auxin responsive factor, (identified by differential display RT-PCR) was examined further for its direct *versus* indirect dependence on *OsMADS1* The detailed expression pattern of this *OsMGH3* was established in a parallel study (Prasad et al , 2005), which showed overlap of its profile with that of *OsMADS1* Using an ectopic but dexamethasone inducible OsMADS1-ΔGR fusion protein (Prasad et al , 2005) we show here that *OsMGH3* expression requires *OsMADS1*, but is regulated indirectly As to start identifying direct targets differentially regulated sequences we have examined the occurrence of the *cis*-regulatory element CArG boxes in forty of these factors The CArG box is the consensus bound by MADS factors and a few of these genes identified here with the CArG motif may be the direct targets of OsMADS1

While this study is exploratory and describes changes in gene expression controlled by *OsMADS1* it is not exhaustive, and was constrained by several technical limitations. Transcripts with low abundance or those with highly localized expression may have been missed Further, the occurrence of CArG motifs in the differentially expressed targets is only indicative, and quite unlikely that these genes are not direct targets of OsMADS1 In fact the sub-set of genes whose expression is affected by loss-of-*OsMADS1* may not be organized within a single network, since other overlapping parallel programs of gene expression controlled by partners of *OsMADS1* need to be examined As yet partners for OsMADS1 are not known In spite of these limitations the differentially regulated genes identified have provided a basis for the further functional analysis of factors that could regulate early aspects of organogenesis and will aid in a mechanistic understanding of *OsMADS1* function

V.2. *Pair-duplicated maize 'C' function genes are divergent with regard to their role in carpel specification –similar duplicate 'C' function genes occur in rice*

In all flowers the position of reproductive-organs stamens and carpel occupy conserved positions. In *Arabidopsis* a single gene that is *AGAMOUS*, controls floral determinacy, carpel fate in the whorl 4, and along with class B patterns stamens in whorl 3 The maize *ZAG1* and *ZMM2*, genes are thought to together constitute the C-function activity Functional analysis of *ZAG1* showed it to control floret determinacy The role played by *ZMM2* in stamen and carpel fate is not known. The divergence and duplication of this

gene-pair is predicted to have occurred ~72 MYA, slightly preceding the large-scale diversification of grasses (Mena et al 1996) To date, in the rice a single gene, *OsMADS3* has been implicated for C-function activity Preliminary studies from other laboratories involving its partial knockdown suggest that *OsMADS3* plays a role in both stamen and carpel formation The maize *ZMM2* show greater sequence identity (82.7%, amino acid identity) to *OsMADS3*, with the rice ortholog for maize *ZAG1* not yet being identified We adopted gain-of-function approaches to study functional diversification among this maize gene pair. Ectopic expression of *ZAG1* caused homeotic transformation of lodicules to carpels, with a malformed depressed palea These data suggest *ZAG1* alone can confer carpel fate in rice spikelet/floret organ development, in addition to its previously known role in conferring floret determinacy in the fourth whorl In contrast we find ectopic expression of *ZMM2* alone does not confer carpel identity, and has only weak effects on male sterility These data suggest functional divergence of *ZAG1* and *ZMM2* The divergence in functional activity might be possibly mediated by the requirement to specific partners for these C-function genes We attempted simultaneous overexpression of both *ZAG1* and *ZMM2* rice, but this seriously affected plant regeneration during tissue culture and therefore transgenic rice could not be generated To explore phenotypes of *OsMADS3* we expressed antisense RNAs for *ZMM2*, or hair-pin precursor RNAs based on *ZMM2* These plants did not have any floret phenotypes most likely indicating inefficient shutdown or the occurrence of a redundant gene in rice We explored the possibility of a duplicated gene through computational analysis of the rice genome We identify, the gene clone (GenBank Accession Number AAT85115) belonging to the C-function clade, in addition to the previously known gene *OsMADS3* We show that expression of AAT85115, now termed *OsAG2*, is limited to stamens and carpels, like *OsMADS3* Through molecular phylogeny and genome synteny we show that maize *ZMM2* is homologous to rice *OsMADS3*, while maize *ZAG1* the homolog of rice *OsAG2*

V.3. *Effects of Arabidopsis SUPERMAN in the cellular control of cell proliferation: Assays in tobacco BY-2 cell lines*

Previous studies with the ectopic expression of *Arabidopsis SUPERMAN* in rice showed its conserved role in control of cell proliferation within floret (Nandi et al , 2000) We have

examined the effects of *SUP* overexpression on vegetative development and find that its overexpression caused juvenile lethality and dwarf plants These phenotypic effects occurred despite any gross change in SAM morphology, indicating subtle changes in sub-populations of SAM cells may underlie the overexpression effects Using tobacco BY-2 cells the general effects of ectopic expression of *SUPERMAN* were examined We find that this resulted in the formation of endoreduplicated cells, and also cause slow progression through G1/S window of the cell cycle Although, the direct the link between entry into differentiation programs is not clear, our preliminary data suggest an inhibitory effect of *SUP* on cell division The results are consistent with the proposed role *SUP* as an inhibitor of cell proliferation, by possibly repressing the transcription of genes that are involved in the activation of cell division (Hiratsu et al , 2002) We speculate that its role in the delimiting the stamen versus carpel boundary between floral whorl 3 and whorl 4 could be thorough restricting excess proliferation of cells that express *AP3*